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13. ABSTRACT (Maximum 200 words) <i>Frederick, MD 21702-5012</i> <p>Leishmaniasis, a disease caused by protozoan parasites of the <u>Leishmania spp.</u>, is one of the major public health problems currently affecting humanity. Therapeutic agents for this disease are either ineffective or toxic. Malaria is considered to be the most important infectious disease of humans on a world-wide scale. It is estimated that 300 million persons are infected at any one time in the world. The purpose of this work is to aid in the development of an effective, non-toxic treatment for leishmaniasis and malaria.</p> <p>The objective of this research was to: 1) Determine the action of the antimalarial 8-aminoquinolines on <u>Leishmania</u> sp., 2) To identify and characterize unique DNA synthetic enzymes for the purpose of chemotherapeutic exploitation and 3) To test potential compounds sent by WRAIR and others for antiparasitic activity.</p>					
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FOREWORD

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Linda Nolan 11/29/95
PI - Signature Date

I. CONTINUATION OF DNA POLYMERASE RESEARCH

Introduction

Leishmaniasis is the second most prevalent disease of protozoan origin, second only to malaria. Two to three million people in tropical and underdeveloped countries are infected annually. The major forms of leishmaniasis are cutaneous, mucosal, and visceral diseases and these result from multiplication of parasites in macrophages of the skin, mucous membrane, and reticuloendothelial system, respectively. The parasites cause severe ulceration in the skin, liver, and spleen, and completely destroy the immune system and bone marrow. Leishmaniasis is fatal if not treated properly. The treatment of choice for all forms of leishmaniasis is very limited. The most common drug available is pentavalent antimony (SbV). The other drugs used commonly are pentamidine and amphotericin B. Drug manufacturers see no economic incentive to manufacture new drugs for these regions of the world. The traditional herbal remedies are left as the best choices for treatment of various parasitic and other diseases in these tropical and underdeveloped countries.

One of our approaches for antileishmamal chemotherapy is selectively targeting the parasite's enzymes, specifically the enzymes involved in DNA synthesis, i.e., DNA polymerases. Many of these enzymes involved in the synthesis of nucleic acids of *Leishmania* have been found to be unique providing a powerful focal point for chemotherapeutic exploitation.

Figure I demonstrates the polymerization of deoxyribonucleotides from deoxyribonucleotide triphosphates in the presence of DNA which functions as primer/template. A typical DNA polymerase reaction involves the presence of a template/primer, substrate, a divalent cation and an -SH group. Blocking any of these components in the enzyme reaction or affecting their binding site to the enzyme or active site of enzyme would inhibit the enzyme activity.

Recently, we have isolated mitochondrial DNA polymerase from *Leishmania mexicana* in order to investigate it as a chemotherapeutic target. Leishmanial mitochondrial DNA polymerase is different from *Leishmania* DNA polymerase A and B. Because of the uniqueness of *Leishmania* DNA polymerases, we proposed that they could be appropriate specific targets of antileishmanial chemotherapy. One of the primary objectives of our research is to better understand the enzymatic mechanism of kinoplast DNA replication in *Leishmania mexicana*, since very little information is available about the enzymatic reactions involved in kinoplast DNA replication. Besides topoisomerase (s), the only homogeneous replicative enzyme that has been shown to be mitochondrial is reported by Tori and England (1992) in *Critchidia fasciculata* (1). We have currently isolated and partially are purifying DNA polymerase activity from mitochondria and are in the process to purify it to near homogeneity. Our results indicate that the activity we have isolated from mitochondria is different than the other two activities previously isolated from the cell extract of the parasitic protozoan *Leishmania mexicana*.

Body

Materials and Methods

Unlabeled nucleoside 5'phosphate and synthetic poly- and oligonucleotides were obtained from P-L-Biochem. [³H]dTTP (specific activity 20.5 Ci/mmole) was obtained from New England Nuclear. Molecular weight markers were purchased as a standard gel filtration calibration kit from Pharmacia. DEAE Sephadex from Sigma; cellulose phosphate and GF/A filter 2.5 cm from Whatman; Bio-Gel HT from Bio-Rad; ssDNA from Pharmacia (LKB Biotechnology, Inc.); centricron- IO micro concentrator was from Amicon (Danvers, MA). All other reagents were purchased from Sigma or Pharmacia. BuPdGTP was a gift from Dr. George Wright (University of Massachusetts Medical Center, Worcester, MA).

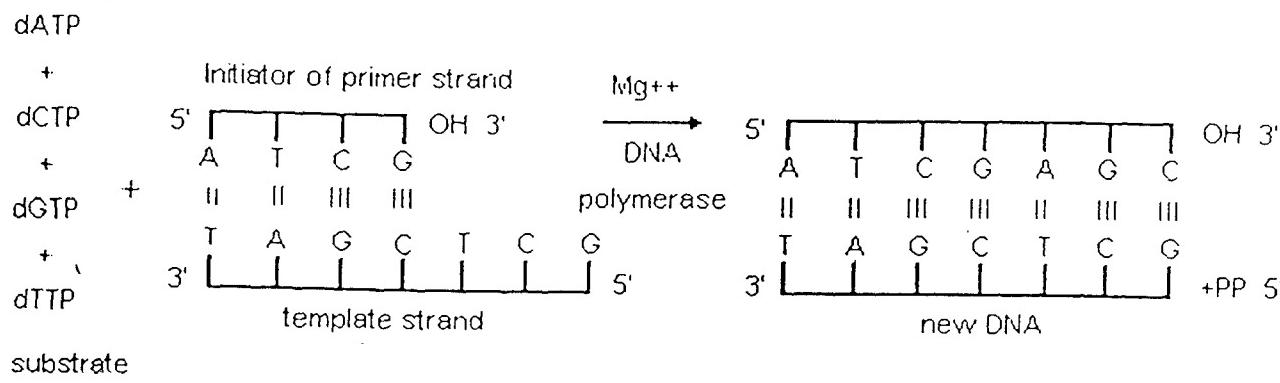


Figure 1: DNA polymerase catalyzes the polymerization of deoxyribonucleotides from deoxyribonucleoside triphosphates in the presence of a DNA which functions as a primer-template. The above reaction is at equilibrium when the substrate (dNTP's) and pyrophosphate (PP_1) are present in approximately the same concentrations.

Antibodies to *Crithidia fasciculata* mitochondrial DNA polymerase raised in rabbits were a generous gift from Drs. Torri and England, (Johns Hopkins University, Baltimore, MD). Anti-human poly a IgG SJK 132-20 was from Pharmacia.

DNA Polymerase Assays

The reactions (25 µl) contained 50 mM Tris-HCl (pH 9.0), 5 mM MgCl₂, 0.1 mg/ml BSA, 2 mg/ml activated calf thymus, 10 µM ³H-TTP (5000 cpm/pmole) 0.05-1.25 units of DNA polymerase and were incubated for 30 minutes at 37°C. The reaction was stopped by adding 1 ml of 10% TCA, 0.1 M Na₄P₂O₇. The samples were collected under vacuum on GF/A filters (Whatman) and washed first with 10 ml 1% TCA, 0.1 M Na₄P₂O₇ and then with 1 ml 95% (v/v) ethanol. The filters were dried under heat lamp and counted by adding 1 ml Bio-Safe II liquid scintillation in a Delta 300 scintillation counter (Searle Analytic, Inc.). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the incorporation of 1 pmole DTNT into the acid-insoluble material in 30 min at 37°C.

Growth of Parasite

Growth of *Leishmania mexicana amazonensis*. Promastigotes of *L. mexicana* Walter Reed Strain were grown as described previously (Nolan, 1987) in the defined medium, brain heart infusion supplemented with 0.2% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY) and 10 mg/ml gentamycin (2).

Buffers

The following buffers were used: *Buffer A*: (DLB-digitonin lysis buffer) 10 mM MOPS (pH 7.5), 0.25 M mannitol, 250 µM MgCl₂, 250 µM EDTA, 5 µM L-ascorbic acid, 0.6 mg/ml polyvinyl pyrrolidone, 0.3 mg/ml BSA, 0.2 mM PMSF,

and 1 µg/ml leupeptin; *Buffer AD*: 0.1 g of digitonin/ml of N,N-dimethylformamide, diluted 1: 10 in buffer A, *Buffer B*: (STE) 250 mM sucrose, 20 mM Tris-HCl (pH 7.5), 20 mM EDTA, 0.2 mM PMSF, 1 µg/ml leupeptin; *Buffer L*: (MLB; mitochondria lysis buffer) 200 MM Tris-HCl (pH 7.8), 20 mM EDTA, 0.5 M KCl, 40 % (v/v) glycerol, 10% (v/v) Nonidet P40, 10 mM DTT, 4 mM PMSF, 20 µg/ml leupeptin; *Buffer C*: (columns buffer) 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 20% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, 1 mM DTT, 0.2 mM PMSF, 1 µg/ml leupeptin; *Buffer CP*: 50 mM KPO₄ (PH7.5), 1 mM EDTA, 2% (v/v) glycerol, 1% (v/v) Nonidet P₄O, 1 mM DTT, 0.2 mM PMSF, 1 µg/ml leupeptin.

Inhibition Assays

Compounds were tested in duplicate by dissolving them in buffer (nucleotides, araCTP, KCl) or in DMSO (aphidicolin, sangivanimycin, berenil) or in ethanol (N-ethylmaleimide, erythromycin) to enzyme assay reactions as appropriate. Control reactions for the inhibitors contained the appropriate volume and concentration of buffer, DMSO, ethanol or non immune rabbit serum.

Enzyme Neutralization Assays

Mitochondrial enzyme activity from *L. mexicana* was incubated at 4°C for 60 minutes in the presence of rabbit antibody raised against mitochondrial DNA polymerase from *C. fasciculata* or in the presence of anti-human IgG SJK 132-20 and assayed as described above. Extra bovine serum albumin was added to the preincubation mixture as to maintain a final protein concentration of 1 mg/ml.

Glycerol Gradient Sedimentation

Linear 10-30%, 4.8 ml gradients containing 50 mM Tris-HCl, pH 7.8, 0.5 M KCl, 1 mM DTT, 0.2 mM PMSF, 1 µg leupeptin were formed in 13 X 51 cm

polypropylene tubes. One ml (fraction V) was concentrated in a Centricon Centrifuge Concentrator (Amicon) to approximately 200 μ l and the buffer was then replaced with the glycerol gradient buffer containing 10% (v/v) glycerol. The sample was then further centrifuged until the final sample was 60 μ l. The concentrated sample was diluted with glycerol gradient centrifugation buffer, minus glycerol to a final glycerol concentration of 10% (v/v), and 100 μ l of this sample was layered onto the gradient. Parallel gradients containing 0.5 mg/ml cytochrom C (1.7 S), BSA (4.31 S), carbonic anhydrase (3.8 S), and catalase (11.1 S) were processed identically. The gradient was centrifuged in a Spino SW50 Ti rotor at 40,000 rpm for 20 hrs at 4°C. Gradients were fractionated from the bottom into 200 μ l aliquots, and polymerase assays were performed immediately. Cytochrome C, catalase and carbonic anhydrase assays were done by reading OD at 405 and 280 mA respectively. The sedimentation coefficient of the polymerase was determined by the method of Martin and Ames (1971) using cytochrome C, BSA, carbonic anhydrase and catalase as the standard references (3).

Protein Assays

The protein concentrations were determined by modification of the Bio-Rad Protein assay. The modified method was performed in 96-well microplates by adding 100 μ l of 40% Bio-Rad dye reagent (which was prepared by diluting 20 ml Bio-Rad Concentrated Dye Reagent (5000-0006) with 30 ml deionized water and filtered through Whatman filter paper) to 100 μ l blank, or BSA or unknown protein as appropriate. The microplate was read at 590 nm using the Softmax 2.32 program designed for Apple Macintosh IIxi Computer System and Molecular Devices ThermoMax Microplate Reader.

Isolation and Partial Purification of Mitochondrial DNA Polymerase

The various stages of purification were performed essentially as by Tori and England (1992). The summary of purification is shown in Table 1. All procedures were performed at 40°C and all columns and dialyses buffers were prepared in buffer C.

Crude Mitochondrial Extract

Wet weight 10 grams of fresh cell pellet of *L. mexicana* 227 promastigotes were suspended at 20°C in 40 ml buffer A, 10 ml of buffer AD was added slowly with gentle mixing to achieve a final concentration of digitonin of 0.1 g/10g of wet cell pellet. The cells were allowed to swell on ice for 2 hours and the extent of swelling was monitored by light microscopy to ensure that almost all cells were intact. After swelling, cells were centrifuged (5000 X g, 10 min, 40°C) and resuspended in 40 ml of buffer A. Using dounce homogenizer fitted with loose pestle (A) and sonicated for 5 s at an output of 65 W on a Braunsonic 2000 Sonicator. The extent of lyses was monitored by light microscope. The lysate was centrifuged (27,000 X g, 30 min, 40°C) and resuspended in 50 ml buffer B and centrifuged again (27,000 X g, 30 min, 40°C). The pellet (crude mitochondria) was resuspended and centrifuged again (35,000 Xg, 30 min, 40°C) to remove all the debris from the mitochondria lysate. The supernatant was dialysed overnight against buffer C containing 0.1 M KCl at 40°C.

DEAE-Sephadex A-25 Chromatography

The dialysed fraction I (centrifuged at 27,000 X g, 30 min, 40°C) was applied to 25 ml packed DEAE-Sephadex column. The column was washed with 50 ml buffer C. This step was essential to remove the nucleic acid as well as of proper

binding of enzyme to phosphocellulose column. The active fractions were pooled and processed to phosphocellulose column.

Phosphocellulose Chromatography

The fraction II obtained from the DEAE-Sepahdex was added directly by gentle stirring to 20 ml phosphocellulose equilibrated in 0.1 M KCl in buffer C (pH 7.8). After 2 hours the slurry was placed in the column and the resin was washed with five column volumes of 0.1 M KCl in buffer C. The enzyme activity was eluted with a stepwise application of 40 ml of 0.2 M KCl, 0.6 M KCl and 0.8 M KCl in buffer C. Most of the enzyme activity was eluted following the 0.2 M KCl step.

Hydroxylapatite Chromatography

The peak fractions (30 ml) of enzyme activity from P-11 chromatography was dialysed overnight against buffer C (pH 7.5) containing 0.5 M KCl and was loaded on (2 ml) Bio-Gel HT column at 0.5 ml/ml which was equilibrated with buffer CP. The column was washed with 10 column volumes of the same buffer. The enzyme was eluted in 0.5 ml fractions using a 20 ml linear gradient from 0.05 to 1 M KPO₄ (pH 7.5). Polymerase activity eluted at 0.30 M and 0.45 M phosphate.

DNA-Cellulose Chromatography

The active fraction from hydroxylapatite column was dialysed against buffer CP and was loaded on 1 ml single stranded DNA cellulose. Washed with 5 ml buffer CP, 0.05 M KPO₄ (pH 7.5). The column was eluted in 0.5 ml fractions with 10 column volumes of a linear gradient from 0 - 1 M KCl in buffer C at 0.5 ml/min flow rate. The enzyme activity was eluted at 0.34 M KCl. The active fractions were pooled and stored at -70°C.

Results

The mitochondrial DNA polymerase was isolated and partially purified as shown in Table 1. Although protease inhibitors were added at all stages of purification, the enzyme activity was very low even after achieving several hundred-fold purification. Crude mitochondria lysate supernatant showed activity just above the background, indicating natural inhibitors in the lysate. Hydroxylapatite chromatography eluted two peaks, one at 0.3 M KCl and the other at 0.45 M KCl. Most of the mitochondrial activity was eluted at 0.3 M KCl. The ssDNA chromatography also revealed a main peak eluted at 0.3 M KCl and a small peak eluted very late, almost at 0.7 M KCl. Because of the low activity of enzyme we were unable to determine the relationship between the two peaks.

Sedimentation analysis of the enzyme carried out in high salt (0.5 M KCl) containing glycerol gradient (10–30 % v/v) glycerol in 50 mM Tris-HCl (pH 7.8), 1 mM DTT and 0.5 mM EDTA revealed a sedimentation co-efficient of 4.25 S when catalase (11.1 S), alcohol dehydrogenase (7.6 S), bovine serum albumin (4.3 S) and carbonic -anhydrase (3.8 S) were used as the protein standards (Fig. 2). Gel exclusion techniques will be used to determine the molecular weight in the future.

Table 2 summarizes the effect of salt and various inhibitors on mitochondria DNA polymerase activity.

Conclusion

Even though all the purification steps were performed in the presence of protein inhibitors, the activity of the enzyme was very low, especially the activity of fraction I was just above the background. Although the activity of enzyme improved after DEAE Sephadex chromatography and a significant increase was noticed after phosphocellulose chromatography, no further purification was carried out after ssDNA column because of lack of total protein as well as lack of stability of

Figure 2

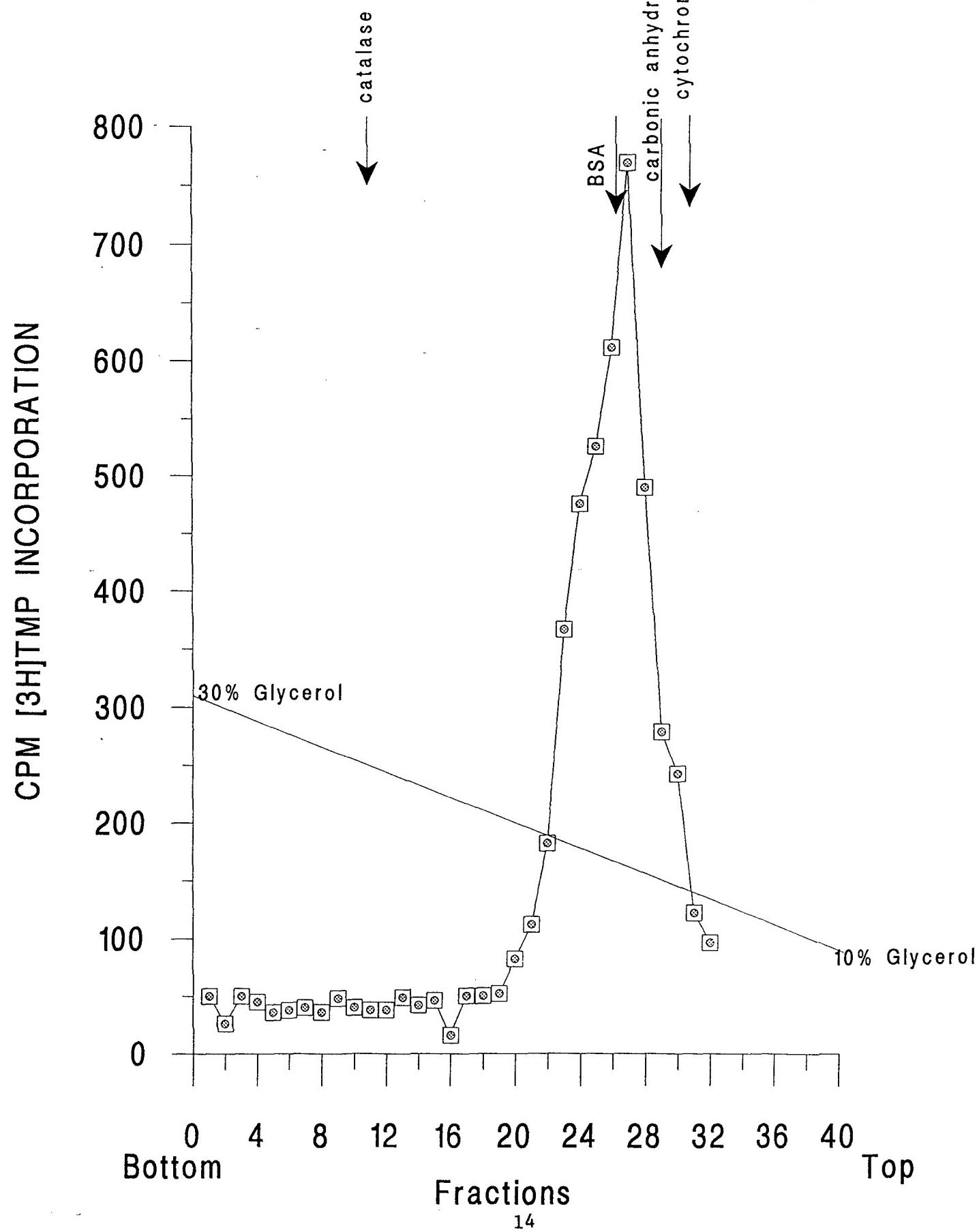


Table 1: Partial purification of Leishmania mexicana DNA polymerase.

Fraction	Purification Step	Total Units	Protein (mg)	Specific Activity U/mg	Purification
1	Mitochondrial lysate supernatant	44.82	287.18	0.15	1
2	DEAE Sephadex	336.8	98.58	3.4	22.6
3	Phosphocellulose	387.0	9.24	41.9	279.3
4	Bio-Gel HT	154.4	2.21	69.5	463
5	ssDNA Cellulose	41.7	0.066	631	4206

1. Specific activity of mix (10 uM $^3\text{HdTTP}$) 5000 cpm/pmoles.
2. $^3\text{HdTTP}$ is the only nucleoside triphosphate used in the assay.

Table 2: Effect of salt, inhibitor, and antibody on mitochondrial DNA polymerase activity.

Reagent	Concentration	% Activity
Control		100
Antibody (a)		
Non immune IgG	10 ug	98
BSA	10 ug	100
Anti-human Pol a IgG 132-20	10 ug	99
Anti- <u>Crithidia</u> mitochondrial DNA polymerase	10 ug	40
Chemical Inhibitors (b)		
dNTP	30 uM	52
KCl	250 mM	6
n-ethylmaeleimide (c)	2 mM	48
BuPdGTP	100 uM	6
Ara CTP	25 uM	59
aphidicolin	100 uM	94
berenil	2 mM	2
erythromycin	2 mM	17
sangivamycin	2 mM	64
dd ATP	100 uM	
dd TTP	100 uM	
dd CTP	100 uM	
dd GTP	100 uM	

enzyme. After this stage 2/3 of the enzyme activity was loaded on glycerol gradient in order to determine the sedimentation profile of mitochondria DNA polymerase. As shown in results, the enzyme sedimented around 4.25 S.

The preliminary experiments show that *Leishmania*'s mitochondrial DNA polymerase activity shows similarity with that of mitochondrial DNA polymerase activity isolated from *Crithidia fasciculata*, since both are inhibited by 200 mM KCl, show sensitivity to NEM (52% inhibition at 2 mM), and are resistant to 100 µM aphidicolin. In addition, addition of other 3dNTP reduce the enzyme activity, a similar observation as noted by Torri and England (1992) in their mitochondrial enzyme activity. Since anti-*Crithidia mitochondrial* shows 60% inhibition to *Leishmania mitochondria* DNA polymerase, this observation indicated further similarity between the two mitochondrial DNA polymerase activities.

We conclude, based on our preliminary data, that the DNA polymerase activity isolated from mitochondrial fraction of *Leishmania* is different than the other two activities Pol A and Pol B which were previously isolated from this protozoan.

II. SCREENING OF NATURAL PRODUCTS FOR ANTILEISHMANIAL CHEMOTHERAPEUTIC POTENTIAL

Introduction

The discovery of effective drugs for the treatment of protozoal diseases infecting mankind represents one of the last unconquered frontiers in drug discovery. Even when significant progress is made, as in the treatment of bacterial infections, resistant, highly pathogenic organisms can appear. Therefore, the search for novel therapeutic agents is imperative. The myriad of structurally diverse compounds found in nature offers a unique source for drug discovery. We have identified unique enzymes (DNA synthetic) in the leishmanial parasite which offer potential targets for chemotherapeutic exploitation with natural products. Several *in vitro* unique radioactive enzyme micro assays were developed and tested for inhibition by known antimicrobial natural products which demonstrated varying degrees of inhibition in our test system.

Four different herbs were chosen for screening antileishmanial activity on mitochondrial DNA polymerase based on their medicinal use (Table 4) (4, 5), as well as their routine consumption by the general population.

Alfalfa (*Medicago sativa L.*) is a deep-rooted perennial, 30–90 cm in height, with cloverlike leaves and violet-blue flowers in loose heads (Fig. 3A). Alfalfa is a widespread escapee from cultivation and is found in fields and along roadsides (5).

Chamomile (*Chamaemelum nobilum L.*) is a low plant, approximately 30 cm in height with pale green, narrowly lanceolate bracts and white to yellowish-white, double or semi-double flowerheads (Fig. 3B). Chamomile has long been cultivated in Europe, North Africa, and North and South America (4).

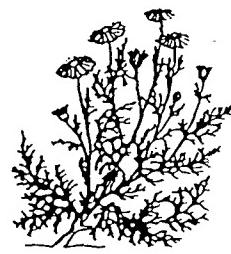
Rosemary (*Rosmarinus officinalis L.*) is an evergreen shrub, approximately 1 m in height, with almost acicular leaves and pale bluish to light bluish violet flowers (Fig. 3C). Rosemary has long been cultivated in Mediterranean countries (4).

Table 3 : Medicinal herbs and their active compounds

Common Name	Scientific Name	Medicinal/Cosmetic Use	Active Compounds
Chamomile	<i>Chamaemelum nobile</i> L. (Aster Family: Compositae)	digestive aid, stomachic antidiarrhetic, sedative antispasmodic, antiemetic analgesic/ hair lightener, skin cleanser and softener	aliphatic (C_4 - C_6) alcohol esters, sesquiterpene lactones and per- oxides, nobilin, epinobilin, flaven- oids (luteolin, apigenin), poly- acetylenes, caffeic & ferulic acids, scopoletin, triterpenes, tiglic & angelic acids, chemazulene, cineole, pinene, pinocarvone, cyclodecane, isobutyrate esters
Alfalfa	<i>Medicago sativa</i> L. (Pea Family: Leguminosae)	antibacterial, antifungal promotes appetite & weight gain, diuretic, estrogenic, anti-inflammatory	tricin, saponins
Rosemary	<i>Rosmarinus officinalis</i> L. (Mint Family: Lamiaceae)	carminative, stomachic rubefacient, liniment antibacterial, antifungal antiprotis/hair darkener, astringent skin lotions	bornol, bornyl acetate, camphor, cineole, pinene, camphene, rosemeric acid
Thyme	<i>Thymus vulgaris</i> L. (Mint Family: Lamiaceae)	rubefacient, carminative counterirritant, antiseptic antitussive/deodorant, healing skin blemishes, toothpaste	thymol, carvacrol, cymene, pinene, bornyl acetate, linalool



A. Alfalfa: *Medicago sativa* L.



B. Chamomile: *Chamaemelum nobile* L.



C. Rosemary: *Rosmarinus officinalis* L.



D. Thyme: *Thymus vulgaris* L.

Figure 3: Herbs used in this study. Sources: alfalfa (8), chamomile, rosemary, and thyme (3).

Thyme (*Thymus vulgaris* L.) is a dwarf shrub with small elliptic leaves and whorls of pale violet flowers (Fig. 3D). Thyme has long been cultivated in Europe, Asia, Africa, and North America (4).

After profiling the *in vivo* activity of the herbal extracts, our next approach was to determine the effect of specific active compounds present in the herbs on *Leishmania* mitochondrial DNA polymerase activity.

Materials and Methods

Cultures of *Leishmania*: *Leishmania mexicana amazonensis* (Walter Reed Strain 227) obtained from the *Leishmania* section of the Walter Reed Army Institute of Research were grown in brain-heart infusion media as previously described (2).

Herbal extracts: Dry alfalfa (*Medicago sativa* L.), chamomile (*Chamaemelum nobile* L.), rosemary (*Rosmarinus officinalis* L.) and thyme (*Aymus vulgaris* L.) and their oils were purchased from a local supermarket, Bread and Circus, Hadley, MA. Each herb was finely ground and 15 ml of 10% DMSO (dimethyl sulfoxide, Sigma Chemical Co., St. Louis, MO) or 80% ethanol (Fisher Scientific, Pittsburgh PA) was added to 3 gm of each herb. The DMSO and ethanol herb slurry was allowed to stand overnight in 25 ml centrifuge tubes, then centrifuged for 10 minutes at 20,000 X g at room temperature. The supernatant was filtered through a 4.5 pm nylon mesh Corning syringe filter and checked for its inhibition on *Leishmania* mitochondrial DNA polymerase activity.

Protein determination: Protein concentrations of the herbal extracts were determined using a bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL).

Active compounds: Thymol and carvacrol were gifts from Dr. K. Shetty (Department of Food Science, University of Massachusetts, Amherst). Alpha-pinene,

camphor, bornyl acetate, saponin, linalool, scopoletin, ferulic acid, caffeic acid, and cineole were purchased from Sigma Chemical Company (St. Louis, MO).

Enzyme assay: One μ l of herbal extract or active compound, at various protein concentrations, was added to a reaction mixture containing 50 mM Tris-HCl (pH 9.0), 5 mM MgCl₂, 0.1 mg/ml BSA, 2 mg/ml activated calf thymus, 10 pM ³H-TTP (5000 cpm/pmole) and 0.1 unit of *Leishmania* mitochondria DNA polymerase to a final volume of 25 μ l, and incubated for 30 min at 37°C. The reaction was stopped by adding 1 ml of 10% TCA, 0.1 M Na₄P₂O₇ and processed as described (6). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the incorporation of 1 pmole dTNW into the acid-insoluble material in 30 min at 37°C.

Results

The herbal extracts showed various degrees of inhibition on *Leishmania* mitochondrial DNA polymerase (Fig. 4–7). The IC₅₀ values (protein concentration) for alfalfa, rosemary, and thyme were determined to be 0.14 mg/ml, 1.10 mg/ml, and 0.82 mg/ml, respectively. No IC₅₀ value was calculated for chamomile since only 36% inhibition of enzyme activity was achieved when the chamomile extract was used at full strength (1.3 mg protein/ml). Fig. 7 shows the comparison of enzyme inhibition at a fixed protein concentration (1.3 mg/ml) by all different herbal extracts. The alfalfa extract almost completely inhibited the enzyme activity at 1.3 mg protein/ml, while the chamomile extract was the least inhibitory. Three grams of herbal extract yielded protein concentrations of 32.8, 23.6, 108.8, and 1.88 mg/ml for chamomile, alfalfa, rosemary, and thyme, respectively.

Since the herbal extracts demonstrated promising results by inhibiting the mitochondrial DNA polymerase activity, the effect of various active compounds (which have been previously demonstrated to possess antimicrobial activity) was also investigated. Low concentrations of the active compounds (obtained commercially) tested showed

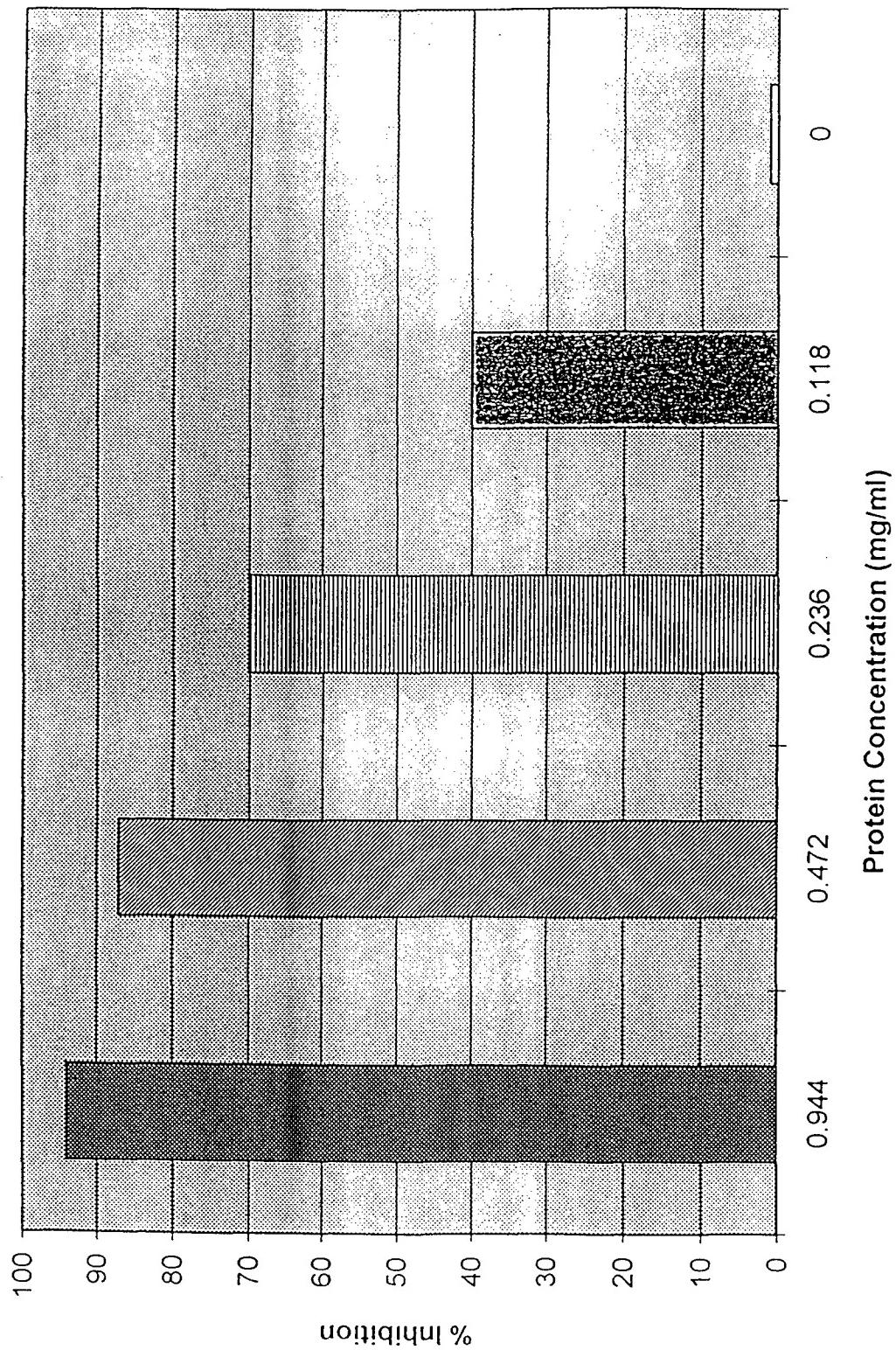


Figure 4: Inhibition of alfalfa extract on *Leishmania* mitochondrial DNA polymerase activity. Enzyme assays were done as described in Materials and Methods.

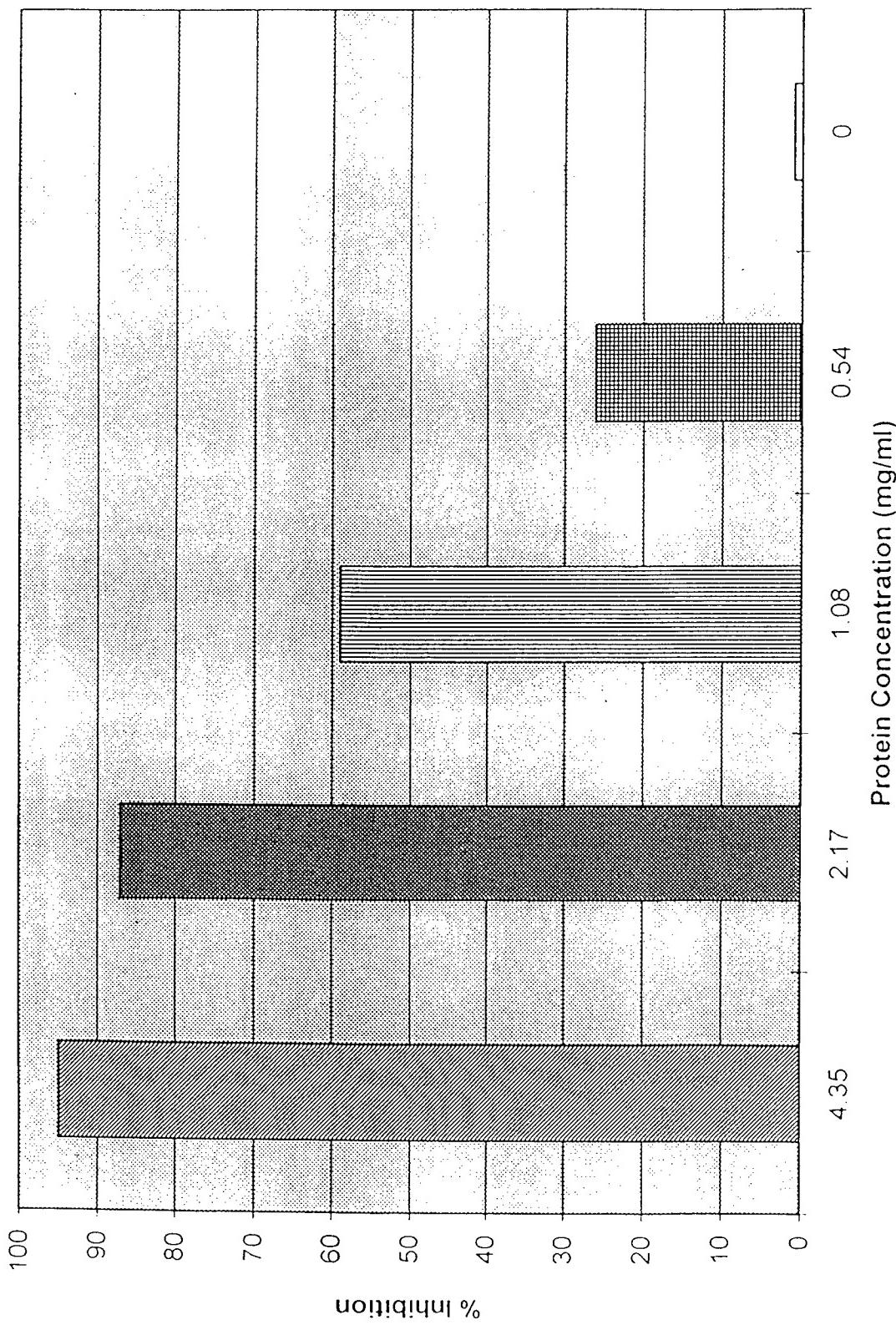


Figure 5. Inhibition of thyme extract on *Leishmania* mitochondrial DNA polymerase activity. Enzyme assays were done as described in Materials and Methods.

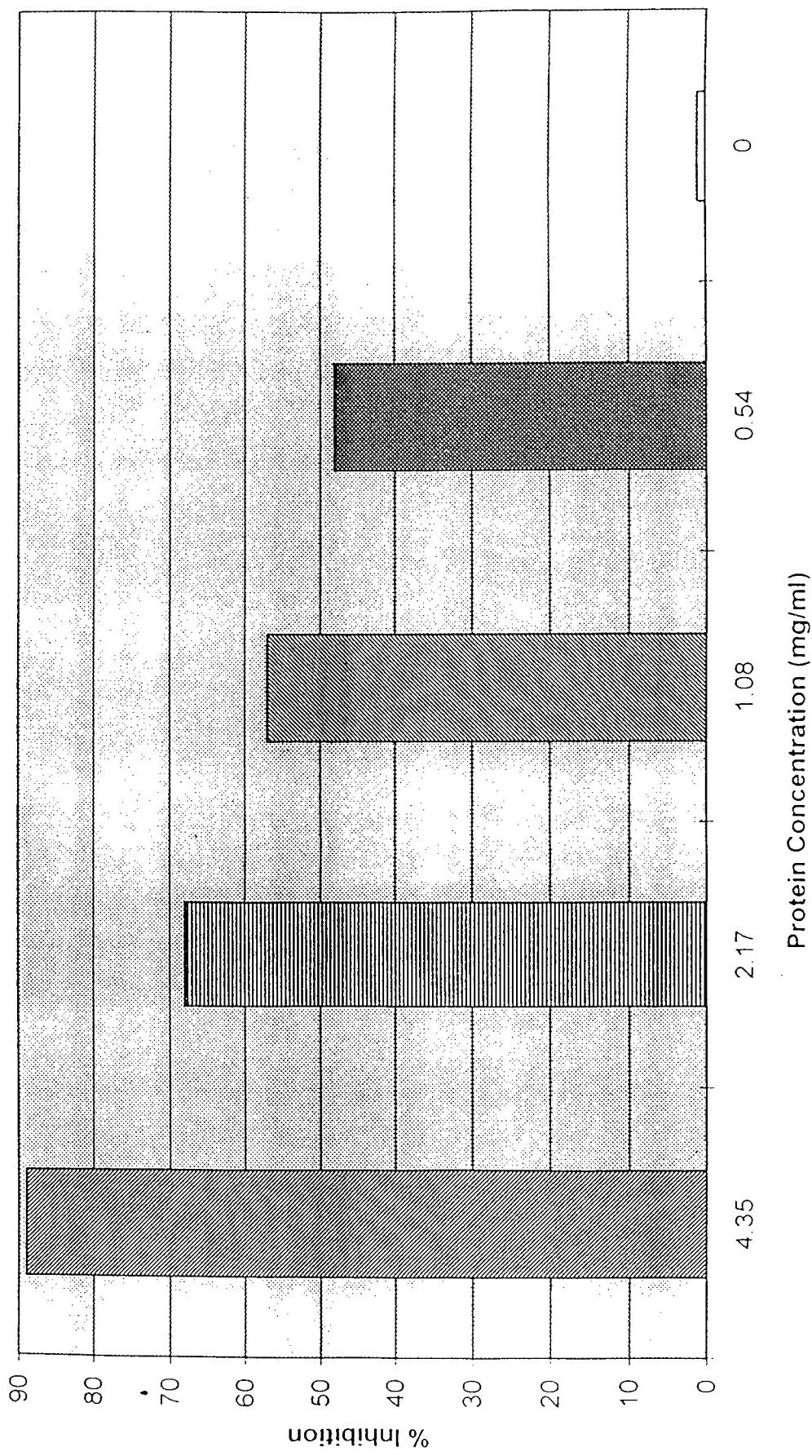


Figure 6. Inhibition of rosemary extract on *Leishmania* mitochondrial DNA polymerase activity. Enzyme assays were done as described in Materials and Methods.

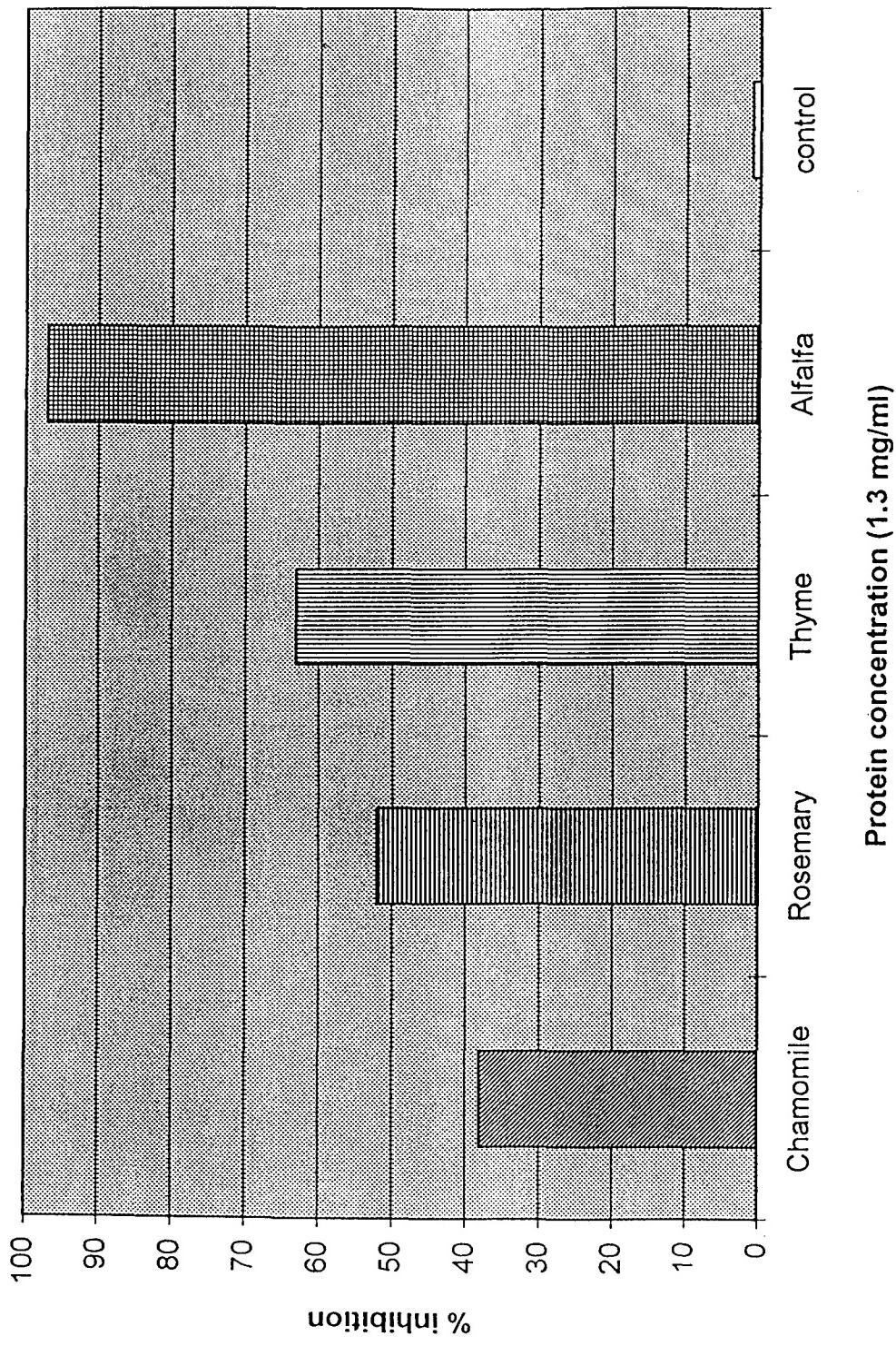


Figure 7: Inhibition of herbal extracts on *Leishmania* mitochondrial DNA polymerase activity. Enzyme assays were done as described in Materials and Methods.

either stimulatory or no significant effect on enzyme activity, except for thymol and carvacrol, which showed 42% and 19% inhibition, respectively. Table 4 presents the results of the various active compounds at 20 mg/ml. None of the active compounds showed any significant inhibition on mitochondrial DNA polymerase enzyme activity. Saponin and ferulic acid showed 29% and 5% inhibition, respectively. The other compounds tested stimulated enzyme activity (Table 4).

The oils of alfalfa, chamomile, rosemary, and thyme had no effect on *Leishmania* mitochondrial DNA polymerase (data not shown).

Conclusion

Our preliminary data indicates that herbal extracts show various degrees of inhibition on DNA polymerase activity isolated from *Leishmania* mitochondria, thus making it a target for antileishmanial chemotherapy. Alfalfa was the most inhibiting on enzyme activity. Thymol demonstrated significant inhibition of activity (72%) at 20 mg/ml. The other active compounds were stimulatory and had no significant effect when tested at various concentrations.

Table 4: Effect of active compounds on *Leishmania* mitochondrial DNA polymerase activity.

Active Compound	Plant Origin	Concentration (mg/ml)	% Activity
Control			100
Cineole	Chamomile, Rosemary	20	123
Caffeic Acid	Chamomile	20	98
Ferulic Acid	Chamomile	20	95
Scopoletin	Chamomile	20	153
Saponin	Alfalfa, Thyme	20	71
Linalool	Thyme	20	116
Camphor	Rosemary	20	99
Bornyl Acetate	Rosemary, Thyme	20	140
Pinene	Chamomile, Rosemary, Thyme	20	96.5
Thymol	Thyme	20	28
Carvacrol	Thyme	20	64

III. SCREENING OF MEDICINAL PLANTS FOR ANTILEISHMANIAL AND ANTIMICROBIAL ACTIVITY

Introduction

Extracts of six plants with known antimicrobial activity were screened for inhibition to *Leishmania mexicana*, a protozoal parasite responsible for a disfiguring disease affecting millions worldwide. Toxic effects were also tested on two mammalian cell lines, CEM T₄ and HeLa. Ethanol, dimethylsulfoxide (DMSO), and aqueous extracts of *Medicago saliva*, *Matricaria chamomilla*, *Rosemarinus officinalis*, *Thymus vulgaris*, *Gaultheria procumbens*, and *Rumex crispus* exhibited varying degrees of inhibition to the cell lines. Inhibition of LM227 was determined by using a microplate assay, measuring absorbance at 660 nm after 24, 48, and 72 h with a microplate reader. Inhibition of the mammalian cell lines was determined by using a test tube assay, measuring absorbance at 660 nm. Ethanol and DMSO extracts exhibited degrees of inhibition against *L. mexicana*, while the aqueous extracts did not significantly inhibit growth. Extract inhibition of the growth of mammalian cell lines was also found. The protein concentration of each extract was tested in order to determine percent inhibition per mg protein. The ethanol and DMSO extracts also caused growth inhibition of several food-borne pathogens and bacteria using a disc assay method.

Medicinal Plants Tested

The plants studied in this experiment were *Medicago sativa* (Alfalfa), *Matricaria chamomilla* (German Chamomile), *Rosemarinus officinalis* (Rosemary), *Thymus vulgaris* (Thyme), *Gaultheria procumbens* (Wintergreen), and *Rumex crispus* (Yellow Dock). These plants, which have a vast array of traditional medicinal uses, were chosen because of their known antimicrobial activity. *M. sativa* extracts have been shown to be effective against Gram-positive bacteria and are antifungal (6). *M. chamomilla* has exhibited both positive and negative bactericidal activity with *Mycobacterium tuberculosis*, *Salmonella typhimurium*, and *Staphylococcus aureus*. Components such as alpha-bisabolol and cyclic ethers are antimicrobial. Umbelliferone is fungistatic, while chamazulen and alpha-bisabolol are antiseptic (6). The oil of *R. officinalis* is bactericidal, fungicidal, and protistcidal. The oil mainly contains δ -pinenes, but also includes camphene, cineole, camphor, and bornyl acid (6, 7). The essential oils of *T. vulgaris* have proven to be active against *Escherichia*, *Salmonella*, and *Streptococcus*. The phenols of the oil may be responsible for their antiseptic effects. One component, Thymol, also acts as a disinfectant as well as a fungicide (6, 7). *G. procumbens* is used as an antiseptic. The known active ingredient is methyl salicylate, the main component of the essential oil (6). *R. crispus* extracts may inhibit *Escherichia*, *Salmonella*, and *Staphylococcus*. Chrysarobin, one of its medicinal compounds, has been used topically for fungal infections, psoriasis, and ringworm. Another compound, rhizome, can also arrest the growth of ringworm and fungi (8).

Materials and Methods

Culture Maintenance

The *L. mexicana* 227 (LM 227) cultures, kept in 75 CM² polystyrene cell culture flasks (Corning), were stored in a standard O₂ incubator at 25°C. The cultures were transferred tri-weekly with Brain Heart Infusion medium (Difco) containing 2.5 ml gentamycin (10 mg/ml), 50 µl hemin, and 3 ml fetal bovine serum (Sigma) per 500 ml media with the ratio of old culture to new serum being 1 to 5.

The T₄ and HeLa cultures were kept in a 5% CO₂ incubator at 37°C. The cultures were transferred tri-weekly with RPMI-1640 media containing 5% fetal bovine serum and 2.5 ml gentamycin (10 mg/ml) per 500 ml medium.

Plant Extracts

Sterile 80% ethanol (EtOH), dimethylsulfoxide (DMSO), and H₂O extracts were prepared from dried plant material obtained at a local supermarket. Three gm of each finely ground plant were added to 15 ml of 80% ETOH, 100% DMSO, or H₂O in 25 ml centrifuge tubes and held overnight at room temperature. The tubes were then centrifuged for 10 min at 10,000 x g and the supernatant fluid was filter-sterilized through a 0.45 µm nylon membrane (Corning).

Protein Assays

The protein concentrations of the extracts were determined using a dye-binding method (Bio-Rad Laboratory, Hercules, CA) using bovine serum albumin (BSA) as a standard. The dye was prepared by diluting 20 ml of concentrated dye reagent (Bio-Rad Laboratories) with 30 ml of H₂O and filtering this solution through Whatman filter paper. One hundred µl of the diluted dye reagent was then added to a 96-well microplate. Each BSA dilution was added in 100µl aliquots to a 96-well

microplate in triplicate with H₂O as the blank. The extracts were diluted 10⁻¹ to 10⁻⁴ with H₂O and 100 µl was added to the microplates. Absorbance (590 nm) was measured after 5 min using a microplate reader (Molecular Devices, Inc.).

LM227 Microplate Assay

The plant extracts were tested with LM227 using 96-well polystyrene Falcon Microtest III tissue culture plates (Becton Dickinson Labware). The culture was first diluted to an absorbance (660 nm) of 0.04 to 0.06 using Steiger and Black medium containing 2.5 ml gentamycin (10 mg/ml), 0.5 ml adenosine (20 mg/ml), 50 µl hemin, and 1 ml of fetal bovine serum per 500 ml media. To all bordering wells of the microplate 300 µl of H₂O was added to provide moisture. The remaining ten rows of six wells each were used for the assay. The blank consisted of 300 µl of Steiger and Black medium. The first control consisted of 300 µl of LM227 culture while the second included 5 µl of extract solvent, and the test consisted of 300 µl LM227 culture plus 5 µl of each plant extract. Each assay was done in at least triplicate. The absorbance (660 nm) of each plate was measured using a microplate reader every 24 h for 96 h (72 h for the water extracts because an arresting of growth was observed). The plates were incubated at 25°C between readings.

Toxicity Assays

Toxicity of the extracts was tested on T₄ and HeLa cell cultures using a test tube assay. The cell suspensions were first diluted with RPMI-1640 medium to yield an absorbance (660 nm) of approximately 0.1. The blank consisted of 5 ml RPMI-1640 medium. Controls consisted of 5 ml of cell culture alone and 5 ml cell culture plus 83 µl of extract solvent. Assay tubes consisted of 5 ml of cell culture plus 83 µl of plant extract in 16 mm x 100 mm Pyrex screw cap test tubes. All assays were

done in duplicate. Test tubes were incubated in a 5% CO₂ incubator at 37°C. Turbidity was measured after 24, 48, and 72 h.

Percent inhibition of the LM227 and mammalian cell lines was calculated by subtracting the 0 hour absorbance then using the formula:

$$[1 - (\text{cell growth of test}/\text{cell growth of control})] \times 100$$

Disc Assay

Lawns of stationary phase cultures of each microorganism were prepared by spread-plating on petri dishes containing nutrient agar. Sterile antibiotic susceptibility discs (6 mm), saturated with 20 µl of each plant extract, were applied to the plates. Zones of inhibition were measured following incubation at 37°C for 48 h.

Results

The extracts with the most effective antileishmanial activity were found to be the 80% ethanol extracts of *M. chamomilla*, *R. crispus*, and *R. officinalis* at protein concentrations of 0.8, 0.8, and 7.5 mg/ml respectively. *M. chamomilla* and *R. crispus* both inhibited the LM227 growth by 100% while *R. officinalis* inhibited the cell growth by 40%. The activity of the different extracts were compared by calculating percent inhibition per mg protein in each ml extract. (Fig. 8) While *M. chamomilla* and *R. crispus* both inhibit LM227 growth 100% at this concentration, *R. officinalis* only inhibits 5% of growth per mg protein. *R. crispus* and *R. officinalis* had no effects on the mammalian cell growth. *M. chamomilla* inhibited HeLa growth 78% and T₄ growth 100%.

The most active DMSO extract was *M. sativa* which inhibited LM227 growth by 43% per mg protein. *R. officinalis* and *G. procumbens* both showed less than 5% activity while *M. chamomilla*, *T. vulgaris*, and *R. crispus* showed no antileishmanial activity. Toxicity of the DMSO alone at a final concentration of 1.7%

was found to be 100% to both the HeLa and the T-4 cell lines and therefore the toxicity of the extracts using this solvent could not be evaluated.

The aqueous extracts showed little inhibition of the LM227 strain. None of the extracts inhibited the growth of LM227 more than 5% per mg protein at 48 h. Because of the relatively insignificant activity of these extracts, their toxicity to was not tested. None of the other extracts studied exhibited antileishmanial activity at 48h.

The DMSO and ethanol extracts were also tested for activity against several bacteria and food-borne pathogens. Growth inhibition of *Pseudomonas aeruginosa*, *Bacillus cereus*, *Shigella dysenteriae*, *Staphylococcus aureus*, and *Bacillus thuringiensis* was observed to varying degrees (Table 5). *M sativa* showed no effect on any of the microorganisms tested. *Escherichia coli* O:157 and the three species of *Salmonella* tested were not affected by any of the extracts.

Discussion

The most promising results discovered in this study were that the ethanol extract of *R. crispus* inhibited the growth of *L. mexicana* 100% and showed no toxic effects on the T-4 or HeLa mammalian cell lines. The ethanol extract of *M. chamomilla* also inhibited the growth of the LM227 culture by 100%. However, it was 78% toxic to the HeLa cell line and 100% toxic to the T₄ cell line. It is quite possible that the toxic effects of the *M. chamomilla* ethanol extract on the mammalian cells is caused by a different compound or combination of compounds than those affecting the LM227 culture. Although not determined, there may be a protein concentration at which the *M. chamomilla* ethanol extract demonstrates leishmanial activity without having toxic effects on mammalian cell lines.

Figure 8. Growth Inhibition of Cell Lines by Ethanol Extracts

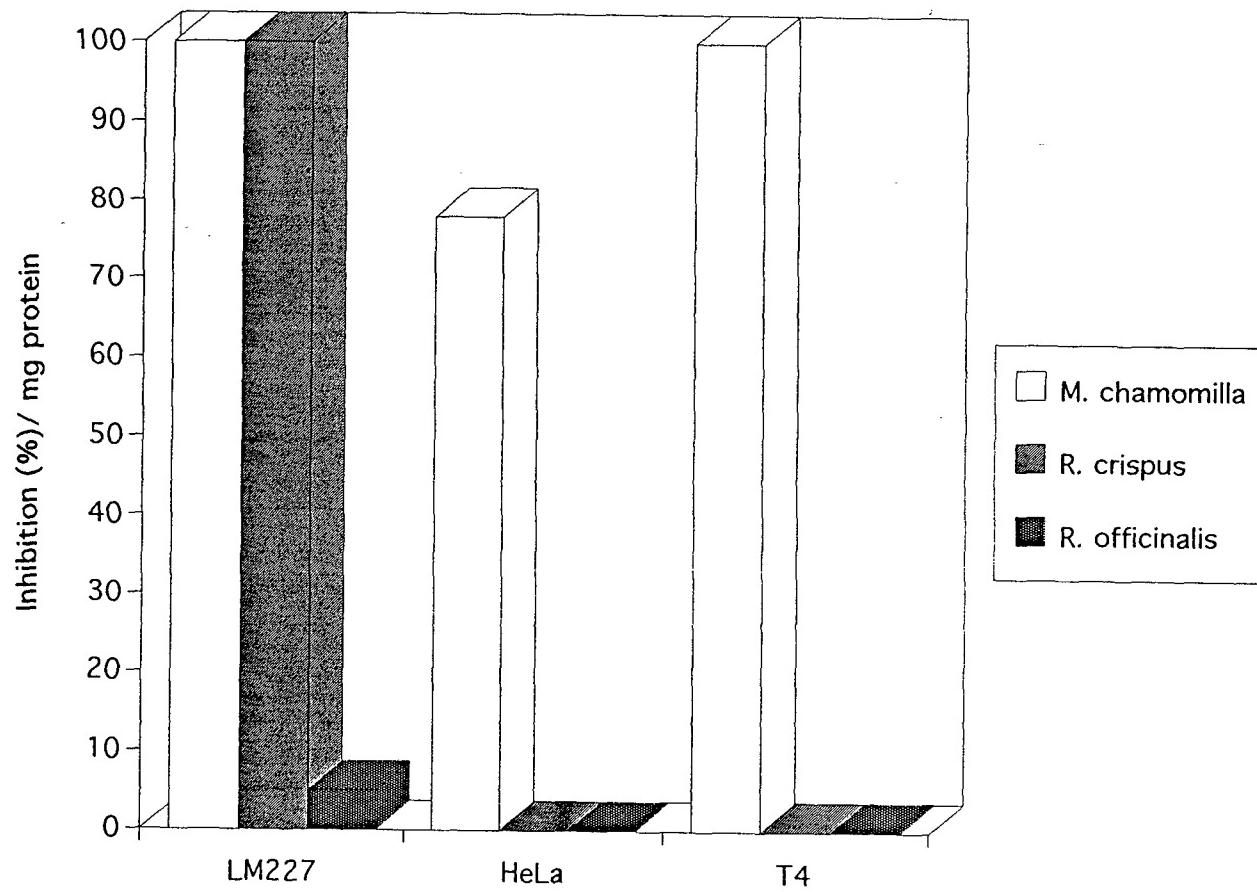


Table 5. Inhibition of Food-Borne Pathogen Growth

	<i>R. officinalis</i>		<i>M. chamomilla</i>		<i>T. vulgaris</i>		<i>R. crispus</i>	
	ETOH	DMSO	ETOH	DMSO	ETOH	DMSO	ETOH	DMSO
<i>P. aeruginosa</i>	-	-	-	-	-	-	+	-
<i>B. cereus</i>	ND	+	ND	+	ND	+	ND	+
<i>S. dysenteriae</i>	-	+	-	-	-	+	-	+
<i>S. aureus</i>	+	ND	-	ND	-	ND	+	ND
<i>B. thuringiensis</i>	ND	+	ND	-	ND	+	ND	+
Protein (mg/ml)	76.8	108.8	8.4	32.8	3.2	108.8	56.0	179.2

ND: Not determined -: No inhibition +: Inhibition

The affects of the *M. sativa* DMSO extract at 43% inhibition also shows promising results even though the toxicity of the potential medicinal compound(s) could not be evaluated. The toxicity of the DMSO on the mammalian cells indicates that an alternate organic solvent must be found to further investigate the medicinal qualities of those compounds affecting the LM227 culture. Ideally, one should find a solvent with reasonably low inhibitory effects on the organism being studied as well as the cells used for toxicity testing.

IV. ANTI-LEISHMANIAL PROPERTIES OF AMAZONIAN PLANT EXTRACTS

Introduction

It has been estimated that 80% of the world's population is dependent upon herbal remedies for the treatment of their ailments and diseases (9). The herbal medicinal traditions of China and India are well known, and people living in developing nations rely almost exclusively on herbal remedies. The success of naturally-derived products in the treatment of ailments has led many scientists to investigate herbal remedies as a possible source of new pharmaceuticals, and natural products have proven to be a rich source of compounds for the production of new medicines. Approximately 50% of the world's leading drugs today contain plant-derived constituents. Pharmacologists use the natural derivatives as templates or precursors for laboratory synthesized compounds. Once isolated from the natural source and purified, chemists may alter the structural features of the biologically active compound to reduce its toxicity or to enhance its medicinal activity. The advantage of modern medicines is in their purity and controlled dosages.

Tropical rainforests are areas of vast floral diversity. Approximately 40% of the world's 250,000 floral species is found only in rainforest regions. Rainforests contain a ten-fold increase in species diversity per unit area over temperate forests. Interest in screening tropical plants has increased in recent years because researchers believe that tropical plants are more likely to yield biologically active compounds than temperate plants. This is due to the intensified competition they face through the increased threat of insect, bacterial, viral and fungal infection they must repel (10). The present urgency for tropical plant products research is due to the rapid depletion of the world's rainforests which at one time covered about 16% of the earth's land surface. This percentage has since been reduced to approximately 7%

of the land surface due to deforestation (11). The 1992 Food and Agriculture Organization (FAO) estimates the annual rate of rainforest destruction at 170,000 square kilometers (12). Naturalists fear that a mass extinction of valuable plant species is inevitable. The eradication of so many species will result in the depletion of genetic diversity and in the loss of future, undiscovered phytopharmaceuticals.

The Waorani Indians of the Amazon rainforest of eastern Ecuador are an isolated tribe whose medicinal practices have been minimally exposed to outside influence. These forest people derive virtually all of their physical, nutritional and medicinal needs from the flora and fauna of the Amazon and are a prime target for ethnobotanical research (13). The Amazon rainforest of eastern Ecuador is one of the most floristically diverse areas of the world (14). Unfortunately, Ecuador has the highest rate of rainforest destruction in South America (12) and the Ecuadorian government has recently signed agreements targeting Waorani lands for petroleum exploration. The pressure of increased contact with the outside world threatens to destroy the Waorani's culture and their herbal medicine traditions.

In August of 1993 a team of researchers including Dr. Linda Nolan (School of Public Health and Health Sciences, University of Massachusetts at Amherst), Dr. F. Abel Ponce de Leon (Veterinary and Animal Sciences, University of Massachusetts at Amherst), Simon Zatyrka (Department of Environmental Health Sciences, University of Massachusetts at Amherst), Dr. John L. Roth (Botanist, Holyoke High School, Holyoke, MA), and Dr. Robert Wallace (University of Florida, Gainesville, FL) traveled to eastern Ecuador to visit the Waorani and to collect medicinal plants. *Funds for travel were obtained from an internal grant from the University of Massachusetts.* Thirty-seven plant specimens were collected under the guidance of Komi, a Waorani herbal healer. Most Waorani medicines are cold water infusions of barks or leaves. Aqueous extracts of the plants have been initially

screened for biological activity against *Leishmania* sp., an infectious protozoan, and against mammalian representative HeLa and CEM T₄ cell lines.

Materials and Methods

Medicinal plants: Table 6 lists the 12 plants selected for toxicity testing, including their medicinal uses and active compounds.

Preparation of plant extracts: A mixture of finely-ground dry plant material and water was ground together with a mortar and pestle for 5–10 min. The supernatant was separated from the pellet by centrifugation (25,000 x g) in a Sorvall RC-5B refrigerated centrifuge (Dupont Instruments, Newton, CT) and sterilized through a Corning 0.45 µm filter (Fischer Scientific, Pittsburgh, PA).

Protein determination: Protein concentrations for the plant extracts were quantified through a modified Bio-Rad dye-binding assay. Assays were conducted in Falcon 3071 microplates (Fischer Scientific, Pittsburgh, PA) and analyzed by a Molecular Devices' THERMOmax microplate reader (Menlo Park, CA) at 590 nm.

Cultures of parasitic protozoa: Promastigotes of *Leishmania mexicana* (Walter Reed Strain 227) and *Leishmania chagasi* (Walter 8 Reed Strain 13) were obtained from Dr. Joan Jackson (Walter Reed Army Institute of Research). The protozoans were grown in defined nutrient media MM2, supplemented with 0.5% gentamycin and incubated at 27°C in an oxygen rich incubator.

Cultures of mammalian cells: HeLa human cervical cells were obtained through the courtesy of Dr. F. Abel Ponce de Leon (Veterinary and Animal Sciences, University of Massachusetts, Amherst). CEM T₄ human leukemic cells were compliments of Dr. Carl Mulder (Department of Pharmacology, University of Massachusetts Medical Center, Worcester). Both cell lines were cultured in RPMI-1640 media supplemented with 5% fetal bovine serum and 0.5% gentamycin, and incubated at 37°C in 5% CO₂.

Table 6 : Medicinal plants selected for toxicity testing.

Waorani	Scientific		
Name	Name	Medicinal Use	Active Compounds
Boyomo	<i>Pentagonia spathicalyx</i>	stingray wounds, infections (18)	unknown
Caca	<i>Bixa orellana</i>	digestive aid, expectorant (18), prussic acid poison antedote	ethereal oils, tannins, saponins, mono- & sesquiterpenes
Ceneiwe	<i>Himatanthus scuuba</i>	warble fly infestations, wounds	alkaloids, cardenolides, polyphenols, terpenes, lignans, coumarins
Noogowe	<i>Hevea guianensis</i>	warble fly infestations, wounds	unknown
Nempocao	<i>Piper augustum</i>	prevents tooth decay & plaque formation, anti-fertility	unknown
Acowe	<i>Leguiminosae sp.</i>	mites, scabies, & fungal infections	unknown
Coonei	<i>Clibadium aspernum</i>	ichthyotoxic agent	unknown
Ayahuasca	<i>Banisteriopsis caapi</i>	narcotic, hallucinogenic agent	unknown

Table 6 continued:

Waorani	Scientific		
Name	Name	Medicinal Use	Active Compounds
Toyoba	<i>Sphaeropteris</i> <i>sp.</i>	toothache remedy	flavenoids, cinnamic acids, sitosterol, lupeol, triterpenes
Biyaye *	unidentified	fevers	unknown
Nuncabo *	unidentified	fevers	unknown
Cuyomin- cayle *	unidentified	cold symptoms	unknown

* Possible anti-leishmanial compounds

Assay inoculum: *Leishmania sp.* and mammalian cells were diluted with fresh media 24 hours prior to testing to ensure log phase growth. The inoculum was standardized at the zero hour with a Spectronic 21 spectrophotometer (Bausch and Lomb, Rochester, NY) at an optical density of 0.5 (660 nm).

Microplate assay protocol: Assays were performed in triplicate in Falcon flat bottom, 96-well, polystyrene tissue culture plates. *In vitro* growth of cells was monitored every 24 hours for a 72 hour period with a Molecular Devices' THERMOmax microplate reader at 590 nm for *Leishmania sp.* and 490 nm for HeLa and CEM T₄ cells.

Measurement of toxicity: Cellular toxicity was measured by the protein concentration of the extracts that resulted in 25 and 50 percent inhibition (IC₂₅, IC₅₀) of *in vitro* cell growth compared to control. The chemotherapeutic potential of the plant extracts was determined by comparing its toxicity to the *Leishmania sp.* with its toxicity to HeLa and CEM T₄ cells.

Results

Table 7 lists the IC₂₅ and IC₅₀ values of the 12 plant extracts. Six extracts (i.e. 'Biyayel', 'Cuyomincalyel', 'Nuncabol', 'Cacal', 'Ceneiwel', and 'Toyobal') displayed potent inhibition of *Leishmania sp.* in culture. These same extracts also showed equal or greater inhibition of both mammalian cell lines.

Conclusion

In conclusion, six of these aqueous extracts have proven to be potent inhibitors of *Leishmania* parasites in culture extracts of 'Biyaye', 'Cuyomincalye', 'Nuncabo', 'Caca' (*Bixa orellana*), 'Ceneiwe' (*Himatanthus scuuba*), and 'Toyoba' (*Sphaeropteris sp.*).

Table 7 : Summary of the IC25 and IC50 values determined for the six most potent plant extracts and cultures of *Leishmania chagasi*, *L. mexicana*, CEM-T4, and HeLa cells.

Inhibitory Protein Concentration in Test Wells ($\mu\text{g/ml}$)							
Waorani Scientific Name	Name	<i>L. chagasi</i>		<i>L. mexicana</i>		CEM-T4	
		IC25	IC50	IC25	IC50	IC25	IC50
Biyaye	unidentified	--	4.6	--	3.9	5.2	--
Caca	<i>Bixa orellana</i>	1.4	--	5.2	--	--	--
Ceneiwe	<i>Himatanthus</i>	--	4.3	--	4.3	--	3.0
	<i>Scuuuba</i>						1.2
Cuyomin-	unidentified	--	8.0	--	22.5	--	16.0
cayle							2.5
Nuncabo	unidentified	1.4	--	1.4	--	2.8	--
Toyoba	<i>Sphaeropteris sp.</i>	1.3	--	2.7	--	2.3	--
							1.4
							--

V. HERB EXTRACTS AS POTENTIAL ANTIPROTOZOAL AGENTS

Background

Various medicinal properties have been ascribed to natural herbs. Leishmanial protozoal parasites and mammalian cell lines whose sensitivity to natural herbs was undetermined were tested for susceptibility to aqueous and ethanol plant extracts including nutmeg (*Myristicaceae sp.*), ginger (*Zingiber officinale*), goldenseal root (*Hydrastis canadensis*), garlic (*Allium sativum*), elephant garlic (*Allium scorodoprasum*), onion (*Allium cepa*) and licorice (*Glycyrrhiza glabra*). Growth of cells of *Leishmania chagasi* 13 and *Leishmania mexicana* 227 was monitored after 72 hr at 590 nm in microwell plates using a microplate reader. HeLa cells were cultured in RPMI-1640 medium with 5% fetal bovine serum. Inhibition of HeLa and *Leishmania* cells was expressed as the IC₅₀ in µg/ml. *L. chagasi* was more sensitive to both types of garlic than *L. mexicana*. Extracts from raw onion did not inhibit growth of any of the cell lines. Licorice (*G. glabra*) inhibited leishmanial parasites, but were not toxic to HeLa cells. All the other extracts showed varying inhibitory activities.

Body

Results

The IC₂₅ and IC₅₀ of the natural herbal extracts towards the individual cell lines are shown in Tables 8–10.

Garlic extracts of *Allium sativum* and *Allium scorodoprasum* exhibited the most inhibitory activity against *Leishmania chagasi* 13. The IC₅₀s were 76 µg protein/ml and 32 µg protein/ml, respectively.

Table 8: Summary of inhibitory activity of natural herb extracts on the growth of *Leishmania chagasi*.

Natural Product	Solvent	Protein Conc. Range ($\mu\text{g}/\text{ml}$)	Percent Inhibition Range	IC25 ($\mu\text{g}/\text{ml}$)	IC50 ($\mu\text{g}/\text{ml}$)
<i>Glycyrrhiza glabra</i> (licorice)	AQ ETOH	15-150 0.26-268	7.3-53.2 16.8-53.9	22 15*	85 240
<i>Hydrastis canadensis</i> (goldenseal root)	AQ ETOH	5-15 0.3-10	0.0-5.5 20.2-33.1	none 314	none none
<i>Zingiber officinale</i> (ginger)	AQ ETOH	15-200 0.2-233	29.3-39.4 15.9-40.4	80 42	none none
<i>Myristacea sp.</i> (nutmeg)	AQ ETOH	1-4 4-408.5	9.2-23.8 12.4-16.8	4.25* none	none none
<i>Allium sativum</i> (garlic)	AQ	12-1200	7.2-90.8	35	76
<i>Allium scorodoprasum</i> (elephant garlic)	AQ	0.3-34	3.1-52.0	12	32
<i>Allium cepa</i> (onion)	AQ	2.2-217	4.6-10.7	none	none

* Estimated

Abbreviations: AQ = aqueous extraction; ETOH = ethanol extraction

Table 9: Summary of inhibitory activity of natural herb extracts on the growth of *Leishmania mexicana*.

Natural Product	Solvent	Protein Conc. Range (μ g/ml)	Percent Inhibition Range	IC25 (μ g/ml)	IC50 (μ g/ml)
<i>Glycyrrhiza glabra</i> (licorice)	AQ ETOH	15-150 0.26-268	26.4-41.7 No Inhibition	6*	none none
<i>Hydrastis canadensis</i> (goldenseal root)	AQ ETOH	5-15 0.3-10	0.0-13.2 No Inhibition	none none	none none
<i>Zingiber officinale</i> (ginger)	AQ ETOH	15-200 0.2-233	27.5-38.5 No Inhibition	14*	none none
<i>Myristacea sp.</i> (nutmeg)	AQ ETOH	1-4 4-408.5	18.7-20.9 1.5-34.8	none 31	none none
<i>Allium sativum</i> (garlic)	AQ	12-1200	0.0-93.0	64	105
<i>Allium scorodoprasum</i> (elephant garlic)	AQ	3-121	0.0-64.0	47	100
<i>Allium cepa</i> (onion)	AQ	2.2-222	No Inhibition	none	none

* Estimated

Abbreviations: AQ = aqueous extraction; ETOH = ethanol extraction

Table 10: Summary of inhibitory activity of natural herb extracts on the growth of mammalian HeLa cells.

Natural Product	Solvent	Protein Conc. Range (μ g/ml)	Percent Inhibition Range	IC25 (μ g/ml)	IC50 (μ g/ml)
<i>Glycyrrhiza glabra</i> (licorice)	AQ ETOH	15-150 2.6-268	No Inhibition 10.0-32.2	none 179	none none
<i>Hydrastis canadensis</i> (goldenseal root)	AQ ETOH	5-15 0.3-30	0.0-30.1 10.0-19.0	13 none	none none
<i>Zingiber officinale</i> (ginger)	AQ ETOH	15-200 0.23-23	2.4-15.0 No Inhibition	none none	none none
<i>Myristacea sp.</i> (nutmeg)	AQ ETOH	1-4 4-408.5	0.0-34.0 No Inhibition	2.7 none	none none
<i>Allium sativum</i> (garlic)	AQ	12-1200	9.2-90.8	34	76
<i>Allium scorodoprasum</i> (elephant garlic)	AQ	0.3-330	0.0-16.9	none	none
<i>Allium cepa</i> (onion)	AQ	0.2-22	0.0-10.7	none	none

* Estimated

Abbreviations: AQ = aqueous extraction; ETOH = ethanol extraction

The *Allium* sp. extracts demonstrated similar inhibition towards *Leishmania mexicana* 227. The IC50 for the *A. sativum* extract was 105 µg protein/ml and the *A. scorodoprasum* extract exhibited an IC50 of 100 µg protein/ml.

The *A. sativum* inhibitory activity was similar for HeLa cells (IC50: 76 µg protein/ml), but the *A. scorodoprasum* was less inhibitory to the HeLa cells (no IC25 recorded).

Only three herbal extracts demonstrated inhibitory properties to *L. mexicana* 227. They were *Glycyrrhiza glabra* (IC25: 6 µg protein/ml) , *Zingiber officinale* (IC25: 14 µg protein/ml) , and ethanol extracts of *Myristacea* sp. (IC25: 31 µg/ml) .

More herbal extracts exhibited inhibition towards *L. chagasi* 13, than *L. mexicana* 227, including both aqueous *G. glabra* (IC50: 85 Ag protein/ml) and ethanol *G. glabra* (IC50: 240 Ag protein/ml) . An IC25 was obtained with ethanol extracts of *Hydrastis canadensis* (IC25: 314 µg protein/ml), aqueous extracts of *Z. officinale* (IC25: 80 µg protein/ml) , ethanol extracts of *Z. officinale* (IC25: 42 µg protein/ml), and aqueous extracts of *Myristacea* sp. (IC25: 42.5 pg protein/ml).

The HeLa cells were less sensitive to the herbs tested. Ethanol extracts of *G. glabra* demonstrated an IC25 of 179 µg protein/ml. Also, aqueous extracts of *H. canadensis* (IC25: 13 µg protein/ml) and of *Myristacea* sp. (IC25: 2.7 µg protein/ml) demonstrated inhibitory activity. Aqueous and ethanol extracts of *Z. officinale*, aqueous extracts of *G. glabra*, ethanol extracts of *H. canadensis*, ethanol extracts of *Myristacea* sp. and *A. cepa* demonstrated no IC25s.

Many extracts demonstrated weak or no inhibitory activity (Table 11). IC25s were not demonstrated towards *L. chagasi* 13 for: aqueous extracts of *G. glabra* and *A. cepa*; ethanol extracts of *Myristacea* sp.; and aqueous extracts of *H. canadensis*. Also, IC25s could not be demonstrated towards *L. mexicana* 227 for: extracts of *A. cepa*, ethanol extracts of *G. glabra*, ethanol and aqueous extracts of *H. canadensis*, and ethanol extracts of *Z. officinale*.

Table 11: Herbs demonstrating weak inhibition against HeLa cells, *Leishmania mexicana*, or *Leishmania chagasi*.

Cells	Herb	Solvent	Protein Conc. Range (μ g/m)	Percent Inhibition Range
HeLa	<i>Glycyrrhiza glabra</i> (licorice)	AQ	15-150	0.0-7.9
	<i>Zingiber officinale</i> (ginger)	AQ	15-200	0.0-6.6
	<i>Myristicaceae sp.</i> (nutmeg)	ETOH	4-40	0.0-5.0
<i>L. mexicana</i>	<i>Allium cepa</i> (onion)	AQ	2.2-223	No Inhibition
	<i>Glycyrrhiza glabra</i> (licorice)	ETOH	15-150	No Inhibition
	<i>Hydrastis canadensis</i> (goldenseal root)	ETOH	0.3-30.4	No Inhibition
	<i>Zingiber officinale</i> (ginger)	ETOH	15-200	No Inhibition
<i>L. chagasi</i>	<i>Hydrastis canadensis</i> (goldenseal root)	AQ	5-15	0.0-5.5

Abbreviations: AQ = aqueous extract; ETOH = ethanol extract

Conclusion

The value of any chemotherapeutic agent lies in its selective toxicity for the host. By comparing the inhibitory activities of the natural herbal extracts and the different cell lines, assessment of antileishmanial potential of the herbs is possible.

Our preliminary results indicate that of all the herbs tested, only *Glycyrrhiza glabra* (licorice) and *Allium scorodoprasum* (Elephant garlic) exhibited *in vitro* potential as antileishmanial agents.

Aqueous *A. scorodoprasum* extracts exhibited an IC₅₀ of 100 µg protein/ml against the *Leishmania mexicana* 227 and an IC₅₀ of 32 µg protein/ml against the *L. chagasi* 13. At the highest concentration tested, little inhibition was observed with the HeLa cells.

Incorporation of garlic in herbal medicines has been documented since the dawn of civilization. *A. scorodoprasum* is a close relative to the better known *A. sativum*. The bulb of *A. scorodoprasum* has similar morphology, but is six times larger on average than *A. sativum* (15). It also has less of an odor and taste than *A. sativum* due to a lower sulfur compound concentration (16). It has been reported that *A. scorodoprasum* acts as an anti-tumor agent as well as an anti-cancer agent (17). *A. scorodoprasum* has also been reported to be effective against typhoid, amoeboid dysentery, and parasitic worms (18). The allium has been classified as an antibacterial, fungicide and a weak antiviral. *A. scorodoprasum* is highly nutritious (19). The use of *A. scorodoprasum* as a combative for malnutrition is due to a high concentration of vitamins and essential amino acids (19). The active ingredient of the *A. scorodoprasum* is thought to be allicin. Allicin is not found in intact garlic cells, but is formed by the enzymatic action of alliin alkyl-sulfenate-lyase (E.C.4.4.1.4) on the non-protein amino acid, S-allylcysteine S-oxide (alliin). The enzyme reaction generates many other secondary products as waste. These include pyruvate, ammonia and allylsulfenic acid (20). Other groups are investigating ajoene, diallyl sulfide, allyl disulfide and diallyl trisulfide as potential active ingredients

(21).

The *G. glabra* extract also possesses significant antileishmanial qualities. Aqueous licorice extracts exhibited an IC₂₅ of 6 µg protein/ml against *L. Mexican* 227 and an IC₂₅ of 22 µg protein/ml and IC₅₀ of 85 µg protein/ml against *L. chagasi* 13. The aqueous extracts demonstrated no significant inhibition against the mammalian HeLa cells. The ethanol extracts demonstrate IC₅₀s at 240 µg protein/ml against only *L. chagasi* 13. These striking differences in IC₅₀s between the cell lines and the parasite indicate their potential as antileishmanial agents.

G. glabra is a known antibacterial and weak antiviral agent. The medicinally active compound of the root combats bronchitis, gastric ulcers and Addison's disease (21). The active ingredient in the compound is a saponin-like glycoside. The terpene, glycyrrhetic acid, is a hemolytically active compound and resembles a steroid. Glycyrrhetic acid is used in the manufacturing of carbenoxolone (23). The compound stimulates the adrenal cortex hormone aldosterone. It also has known estrogenic effects (24).

Fractionation of extracts of *G. glabra* and *A. scorodoprasuw* are planned to identify the active inhibitory compounds.

Future Directions

Because of internal prioritization of tropical disease research within WRAIR, this laboratory has been advised by co-technical representative Major Dennis Kyle to begin studies on finding chemotherapeutic targets for the treatment of malaria.

The P.I. and her staff visited Dr. Wirth, an expert in research on malaria, at the Harvard School of Public Health, and we are in the process of turning our efforts to determining the antimarial mode of action of antimarial compounds.

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PHYLLIS M. RINEHART
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